SUSHI PEPTIDE MULTIMER

REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of Canadian Patent Application No. 2,432,972, filed July 4, 2003, the content of which is herein incorporated by reference.

FIELD OF INVENTION

The present invention relates to Gram-negative bacterial infection and peptides which inhibit lipopolysaccharide (LPS)-induced activities.

BACKGROUND

Sepsis remains a leading cause of death in critical care unit, and is also frequently associated with serious consequence such as multiple organ failure. Gram-negative bacterial endotoxin, also known as lipopolysaccharide (LPS), has been suggested to play a pivotal role in such septic complications (Houdijk *et al*, 1997). The acute phase plasma protein, LPS binding protein (LBP), binds circulating LPS to extract it from micelles, and transfer it to either soluble or membrane-bound CD14 receptor in monocytes and macrophages. The interaction of this complex with Toll-like receptors (TLRs) is thought to initiate intracellular signaling reactions, via transcription factor NF-κB. Activation of protein kinases mediates the production of inflammatory cytokines, which contribute to septic shock. It has also been shown that in the absence of plasma LBP, the LPS is able to directly interact with CD14, yielding similar effects. Thus, treatment of endotoxaemia and sepsis would be greatly aided by blocking the activity of endotoxin and/or removing them

from the body fluids of patients, as cationic peptides and analogues do (de Haas et al, 1998; Scott *et al*, 2000).

LPS from gram-negative bacteria induces the amoebocytes of limulus to aggregate and degranulate. This response underlies the important defense mechanism of limulus against invasion of gram-negative bacteria (Ding *et al*, 1995). As a molecular biosensor, Factor C can be autocatalytically activated by femtograms of LPS to trigger the coagulation cascade (Ho, 1983), suggesting that it contains high affinity LPS-binding domains. Recently, two regions of Factor C that exhibit exceptionally high LPS binding affinity were defined as the Sushi1 and Sushi3 domains (Tan *et al*, 2000a). Two 34-mer chemically synthesized peptides, S1 and S3, spanning the 171-204 and 268-301 amino acid residues of Factor C (GenBank Accession No. S77063), are derived from Sushi1 and Sushi3 domains, respectively. (The S3 peptide consisting of residues 268-301 of Factor C is shown in SEQ ID NO:1). Both peptides inhibit LPS-induced limulus amoebocyte lysate (LAL) reaction and LPS-induced hTNF-α secretion (Tan *et al*, 2000b). See also US patent 6,719,973, the entire content of which is herein incorporated by reference. The application value of these two peptides would be boosted if they could be obtained by cost effective and large-scale methods such as recombinant expression in prokaryotic systems. However, expression of smaller peptides tends to encounter technical difficulties (Le *et al*, 1991; Latham, 1999).

Trace levels of endotoxin or lipopolysaccharides (LPS) cause pathophysiological reactions such as fever, changes in white blood cell counts, disseminated intravascular coagulation, hypotension, shock and death. Intensive research is being carried out to develop more

sensitive techniques that are able to remove minute levels of endotoxin from pharmaceutical fluids to meet higher standards of safety (Petsch 2000). Adsorption methods have proven to be the most effective (Minobe 1982) in removing endotoxins from solutions and many methods have been developed for different target solutions with varying efficiencies. However, most of these methods are not efficient over a wide range of pH and ionic strength (Petsch 2000). In addition, there is always a compromise between protein recovery and LPS removal, such that the clearance factor is often disappointing when the LPS feed concentration is low.

SUMMARY OF THE INVENTION

In one aspect, there is described a polypeptide comprising more than one S3 peptides, in particular those peptides in tandem repeat. An example of this is rS3-4mer (SEQ ID NO:9). In specific embodiments, the polypeptide comprises 2 to 10 S3 peptides.

In another aspect, the polypeptide of the invention has at least two of the S3 peptides separated by a linking sequence which may or may not be cleavable. In specific embodiments, the linking sequence is cleavable by protease or by acid digestion. However, not all the linking sequences in the polypeptide need be cleavable. In specific embodiments, the linking sequence comprises Asp-Pro.

In another aspect, the polypeptide of the invention consists of more than one S3 peptides, in particular those peptides in tandem repeat. In specific embodiments, the polypeptide consists 2 to 10 S3 peptides.

In another aspect, there is described the polypeptide of the invention, or S3 peptide, tagged with a detectable label. In certain embodiments, the label is detectable by fluorescence.

In another aspect, there are described DNA encoding the polypeptide of the invention, expression cassettes comprising the DNA of the invention, and vectors comprising the expression cassette of the invention.

In another aspect, there is described a host cell comprising the DNA of the invention.

In another aspect, there is described a method of producing a multimer of S3 peptide, comprising the step of expressing DNA encoding the polypeptide of the invention in a host cell. The method may further comprise the step of isolating the polypeptide.

In another aspect, there is described a method of producing a polypeptide having a desired number of S3 peptides. The method comprises the step of expressing in a host cell DNA which encodes S3 peptides in a single open reading frame, wherein the S3 peptides appear in the open reading frame in greater number than the desired number, and wherein at least two of the S3 peptides are separated by a cleavable linking sequence. The expressed polypeptide is then subjected to conditions suitable for cleaving the linking sequence to produce the polypeptide having the desired number of S3 peptides, but keeping the S3 peptides intact. The method may further comprise the step of isolating the polypeptide having the desired number of S3 peptides from the remaining cleavage products. In some embodiments, the cleavage method may be acid digestion or proteolytic digestion.

In another aspect, there is described a method of producing a polypeptide having four S3 peptides. The method comprises the step of expressing in a host cell DNA which encodes eight S3 peptides in a single open reading frame. There is at least one cleavable linking sequence which occurs between the fourth and fifth S3 peptide sequence. The expressed polypeptide is then subjected to conditions suitable for cleaving the linking sequence to produce the tetramer from the octamer, but keeping the tetramer intact. If necessary, the method may further comprise the step of isolating the tetramer from the remaining cleavage products such as monomer and uncleaved octamer. In some embodiments, the cleavage method may be acid digestion or proteolytic digestion.

In another aspect, there is described a method for detecting LPS-containing bacteria comprising the steps of contacting a sample to be tested for LPS-containing bacteria, with the polypeptide of the invention or S3 peptide and detecting binding between LPS and the polypeptide. The polypeptide of the invention or S3 peptide may be tagged with a detectable label such as a fluorescent tag.

In another aspect, there is described a method for treating endotoxaemia or sepsis comprising the step of administering the polypeptide of the invention to a patient suffering from endotoxaemia or sepsis.

In another aspect, there is described a method for detecting LPS-containing bacteria comprising the step of contacting a sample containing LPS-containing bacteria with the polypeptide or S3 peptide, wherein the polypeptide or S3 peptide is fluorescently labeled, and detecting bacteria-associated fluorescence arising from the label.

In another aspect, there is described S3 peptide or a polypeptide of the invention immobilized on a solid medium such as agarose beads.

In another aspect, there is described a method for removing LPS or LPS-containing bacteria from a sample, comprising the step of contacting the sample with the immobilized polypeptide or peptide of the invention under conditions which allow binding of LPS-containing bacteria to the polypeptide or the peptide, and obtaining the unbound material which is substantially free of LPS or LPS-containing bacteria.

The invention further encompasses commercial packages comprising the polypeptide of the invention and instructions for its use in detecting LPS-containing bacteria in a sample; or instructions for its use in treating endotoxaemia or sepsis.

The invention further encompasses commercial packages comprising S3 peptide or the polypeptide of the invention immobilized on a solid medium, and instructions for its use for removing LPS or LPS-containing bacteria from a sample.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a schematic representation of the multimerization of S3 gene using the gene amplification vector, pBC. The *Bbs*I site was introduced into the S3 primers, and the amplified gene was cloned into pBC vector. After the *Bbs*I digestion, the S3 gene with overhang terminals were self-ligated at 16°C for 2 h, and inserted into pBC which was previously linearised with *BbsI*. The CCCC head motif on the sense strand and GGGG tail motif on

anti-sense strand allowed the fragments to self-ligate directionally, giving rise to multimers of pBCS3-nMer constructs. These multimeric inserts were subsequently released and recloned into expression vector pET22b.

Figure 2 shows an identification of multimers of S3 gene and expression in *E. coli*. (a) Electrophoretic analysis of the multimeric S3 genes. The number of S3 inserts cloned in the pBC was determined by digestion with *Nde*I and *Hind*III, which flank the multimers. The digests were resolved on 2% agarose gel. Lane M, 100 bp DNA ladder; lanes 1-4, *Nde*I and *Hind*III digested pBCS3-1, -2, -4, -8mer, which contain 1, 2, 4, 8 copies of S3 gene. (b) Expression of multimers of S3 gene in *E. coli* BL21. The recombinant peptides were resolved on SDS-PAGE constituting 5% stacking gel and 18% resolving gel. Lane M, peptide markers; lane 1, BL21 containing pET22b; lanes 2-5, BL21 containing S3-1, -2, -4, -8mer, respectively; lane 6, purified rS3-4mer. The arrows indicate the recombinant proteins.

Figure 3 shows a time course of formic acid cleavage of rS3-4mer into monomers and Western blot analysis of recombinant peptides. (a) Digestion of rS3-4mer into monomers. The rS3-4mer was dissolved in cleavage buffer and incubated at 42°C with constant and gentle shaking. At 12, 24, 36 and 48 h, aliquots of 100 µl of samples were sampled and added to 900 µl of ethanol, chilled at -20°C for 30 min, centrifuged at 15000g for 10 min, and dissolved in loading buffer for electrophoretic resolution on tricine SDS-PAGE with 5% stacking gel and 15% resolving gel. Lanes 1-4 are samples digested for 12, 24, 36, 48 h, respectively; lane 5, intact rS3-4mer. (b) Western blot analysis of recombinant peptides. Lane 1, total expressed cell proteins. The expressed 18.4 kDa rS3-4mer strongly reacts with anti-S3

antibody; lane 2, partially digested peptide mixtures containing rS3-1, -2, -3, -4mer ; lane 3, rS3-1mer derived from the rS3-4mer; lane 4, chemically synthesized S3 peptide. All peptides derived from rS3-4mer reacted with the antibody.

Figure 4 shows an ELISA-based LPS binding assay. LPS was coated overnight on 96-well plates. Varying concentrations of peptides were allowed to interact with the immobilized LPS. The amount of bound peptides was determined by rabbit anti-S3 IgG and quantitated by ABTS substrate. The average OD_{405nm} of the triplicate samples were calculated and plotted with the corresponding concentration.

Figure 5 shows a comparison of rS3-4mer and -1mer with chemically synthesized S3 in inhibition of LPS-induced LAL assay and hTNF-α secretion in human THP-1 cells. (a) Inhibition of LPS-induced LAL assay. Binding of the peptides to LPS would competitively inhibit the chromogenic reaction in kinetic-QCL LAL test. The ENC_{50} values of rS3-4, rS3-1mer and chemically synthesized S3 peptide were determined to be 5.4 µg/ml, 9.2 µg/ml, 10.2 µg/ml, respectively. (b) Suppression of LPS-induced hTNF-α secretion in human THP-1 cells. The rS3-4mer and rS3-1mer were tested for their ability to suppress LPS-induced hTNF-α secretion from THP.1 cells. Both peptides inhibit hTNF-α production in a dose-dependent manner, albeit with different efficiency. rS3-4mer required only 40.4 µg/ml to achieve ENC_{50}, compared to 83.2 µg/ml needed for rS3-1mer. The decrease in TNF-α secretion was expressed as percentage of control (LPS only).

DETAILED DESCRIPTION OF EMBODIMENTS

Before describing the present invention in further detail, it is to be understood that this invention is not limited to particular formulations or process parameters as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments of the invention only, and is not intended to be limiting.

Although a number of compositions and methods similar or equivalent to those described herein can be used in the practice of the present invention, the preferred materials and methods are described herein.

S3 has been shown to be one of the LPS-binding sites of Factor C, and is able to suppress the LPS-induced cytokine production in macrophages (Tan *et al*, 2000b). The immobilized S3 peptide analogue can remove LPS from culture medium with high efficiency (Ding *et al*, 2001). Thus, this promising reagent can be applied to prevent sepsis due to circulating LPS, which is released by viable or injured Gram-negative bacteria. Chemical synthesis may be used to obtain large quantity of this peptide, but expression in *E. coli* may be more cost-effective (Latham, 1999). However, the yield from *E. coli* may be low and unstable (Le *et al*, 1991). Thus, expression of the multimers of peptides would circumvent the abovementioned problems (Kajino *et al*, 2000). A more important attribute for recombinant multimers of S3 is the expected enhancement in ligand-binding affinity and LPS-neutralisation activity achieved through synergistic effects of multiple LPS-binding units in one molecule (Mauro *et al*, 2000).

The term "isolated polynucleotide" or "isolated polypeptide" is defined as one which is removed from the environment in which it naturally occurs. For example, a naturally-occurring DNA molecule present in the genome of a living bacteria or as part of a gene bank is not isolated, but the same molecule separated from the remaining part of the bacterial genome, as a result of, *e.g.*, a cloning event (amplification), is isolated. Typically, an isolated DNA molecule is free from DNA regions (*e.g.*, coding regions) with which it is immediately contiguous at the 5' or 3' end, in the naturally occurring genome. Such isolated polynucleotides may be part of a vector or a composition and still be defined as isolated in that such a vector or composition is not part of the natural environment of such polynucleotide.

Many methods can be applied to construct multimers including tandem repeats of a peptide (Lee *et al*, 2000; Mauro, *et al*, 2000; Dolby, *et al*, 1999). Chemical synthesis, especially solid-phase synthesis may be used for short (e.g., less than 50 residues) peptides or those containing unnatural or unusual amino acids such as D-Tyr, ornithine, amino-adipic acid, and the like. Recombinant procedures are preferred for longer polypeptides.

Peptides can be synthesized chemically by such commonly used methods as t-BOC or FMOC protection of alpha-amino groups. Both methods involve stepwise syntheses whereby a single amino acid is added at each step starting from the carboxyl-terminus of the peptide (See, Coligan et al., Current Protocols in Immunology, Wiley Interscience, 1991, Unit 9). Peptides of the invention can also be synthesized by the solid phase peptide synthesis methods well known in the art. (Merrifield, J. Am. Chem. Soc.,

85:2149, 1962), and Stewart and Young, Solid Phase Peptides Synthesis, Pierce, Rockford, Ill. (1984)). Peptides can be synthesized using a copoly(styrene-divinylbenzene) containing 0.1-1.0 mMol amines/g polymer. On completion of chemical synthesis, the peptides can be deprotected and cleaved from the polymer by treatment with liquid HF-10% anisole for about 0.25 to 1 hour at 0°C. After evaporation of the reagents, the peptides are extracted from the polymer with 1% acetic acid solution which is then lyophilized to yield the crude material.

The crude material can typically be purified by such techniques as gel filtration on Sephadex G-15 using 5% acetic acid as a solvent, by high pressure liquid chromatography, and the like. Lyophilization of appropriate fractions of the column will yield the homogeneous peptide or peptide derivatives, which can then be characterized by such standard techniques as amino acid analysis, thin layer chromatography, high performance liquid chromatography, ultraviolet absorption spectroscopy, molar rotation, solubility, and assessed by the solid phase Edman degradation (see e.g. Protein Purification, M. P. Deutscher, ed. Methods in Enzymology, Vol 182, Academic Press, 1990). Automated synthesis using FMOC solid phase synthetic methods can be achieved using an automated peptide synthesizer (Model 432A, Applied Biosystems, Inc.). Peptides are oxidized in Trix-DMSO-isopropanol at pH 7.5 for about 15 to about 20 hours at about 23°C to permit the formation of disulphide bonds (Tam et al. (1991) J. Am. Chem. Soc 113:6657-6662). Following disulphide bond formation, the suspension is purified using reverse phase FPLC by which a yield of about 20% can be obtained.

The invention encompasses recombinant methods for producing a polypeptide comprising a multimer of S3. Such methods comprise the step of introducing DNA encoding the polypeptide into a host cell. If desired, the S3 multimer may be cleaved from the polypeptide.

"Amino acid cleavage site" refers to an amino acid or amino acids that serve as a recognition site for a chemical or enzymatic reaction such that the peptide chain is cleaved at that site by the chemical agent or enzyme. Amino acid cleavage sites include those at aspartic acid-proline (Asp-Pro), methionine (Met), tryptophan (Trp) or glutamic acid (Glu). "Acid-sensitive amino acid cleavage site" as used herein refers to an amino acid or amino acids that serve as a recognition site such that the peptide chain is cleaved at that site by acid. Particularly preferred is the Asp-Pro cleavage site which may be cleaved between Asp and Pro by acid hydrolysis.

Polypeptides containing the multimers of S3 may contain a linking amino acid or amino acids for cleaving the specific multimer of interest from the polypeptide. For example, a polypeptide having multiples of S3 tetramers may contain amino acid cleavage sites between the tetramers. In another embodiment, the desired S3 multimer may be produced as a fusion protein where the S3 multimer is fused to a heterologous polypeptide such as the commercially available His-tag, and where an amino acid cleavage site is placed between the S3 multimer and the heterologous polypeptide. The linking amino acid or amino acids are incorporated between the multimer of interest and the remainder of the polypeptide in such a way that one or more cleavage reactions separate each polypeptide species to the degree necessary for intended applications. In some embodiments,

the linking sequence consists of one or more amino acids, up to 100 amino acids, preferably 1, 2, 3, 4, 8, 10 amino acids. It may not in every instance be necessary to cleave all, some, or any of the species within a particular polypeptide.

As used herein, a fusion polypeptide is one that contains a multimer of S3 fused at the N- or C-terminal end to a polypeptide unrelated to S3, i.e. a heterologous polypeptide. A simple way to obtain such a fusion polypeptide is by translation of an in-frame fusion of the polynucleotide sequences, *i.e.*, a hybrid gene. The hybrid gene encoding the fusion polypeptide is inserted into an expression vector which is used to transform or transfect a host cell. Alternatively, the polynucleotide sequence encoding the S3 multimer is inserted into an expression vector in which the polynucleotide encoding the heterologous polypeptide is already present. Such vectors and instructions for their use are commercially available, e.g. the pMal-c2 or pMal-p2 system from New England Biolabs, in which the heterologous polypeptide is a maltose binding protein, the glutathione-S-transferase system of Pharmacia, or the His-Tag system available from Novagen. These and other expression systems provide convenient means for further purification of the desired S3 multimer.

Amino acids that may be used to link the S3 multimer of interest to the remainder of the polypeptide include aspartic acid-proline, asparagine-glycine, methionine, cysteine, lysine-proline, arginine-proline, isoleucine-glutamic acid-glycine-arginine, and the like. Cleavage may be effected by exposure to the appropriate chemical reagent or cleaving enzyme.

It should be recognized that cleavage may not be necessary for every multimer or fusion polypeptide that is constructed. A cleavage site could be incorporated, or absent.

The invention also encompasses a method of producing a desired S3 multimer of high purity comprising the steps of transforming a compatible host with a vector suitable for expressing a fusion polypeptide containing the S3 multimer, culturing the host, isolating the fusion polypeptide by selective binding to an affinity matrix such as a carrier linked to an antibody specific for the heterologous polypeptide, and cleaving off the desired S3 multimer either directly from the carrier-bound fusion polypeptide or after desorption from the carrier.

A necessary condition to permit such cleavage of the produced polypeptide is that it contains a unique cleavage site which may be recognized and cleaved by suitable means. Such a cleavage site may be a unique amino-acid sequence recognizable by chemical or enzymatic means and located between the desired portion of the polypeptide and remainder of the fusion polypeptide to be produced. Such a specific amino acid sequence must not occur within the desired portion.

Examples of enzymatic agents include proteases, such as collagenase, which in some cases recognizes the amino acid sequence NH_2 --Pro--X--Gly--Pro--COOH, wherein X is an arbitrary amino acid residue, e.g. leucine; chymosin (rennin), which cleaves the Met-Phe bond; kallikrein B, which cleaves on the carboxyl side of Arg in X--Phe--Arg--Y; enterokinase, which recognizes the sequence X--$(Asp)_n$ --Lys--Y, wherein n=2-4, and cleaves it on the carboxyl side of Lys; thrombin which cleaves at specific arginyl bonds.

Examples of chemical agents include cyanogen bromide (CNBr), which cleaves after Met; hydroxylamine, which cleaves the Asn-Z bond, wherein Z may be Gly, Leu or Ala; formic acid, which in high concentration (about 70%) specifically cleaves Asp-Pro. Thus, if the desired portion does not contain any methionine sequences, the cleavage site may be a methionine group which can be selectively cleaved by cyanogen bromide. Chemical cleaving agents may be preferred in certain cases because protease recognition sequences may be sterically hindered in the produced polypeptide.

The techniques for introducing DNA sequences coding for such amino acid cleavage sites into the DNA sequence coding for the polypeptide are well-known in the art.

As mentioned above, cleavage may be effected either with the fusion polypeptide bound to the affinity matrix or after desorption therefrom. A batch-wise procedure may be carried out as follows. The carrier having the fusion polypeptide bound thereto, e.g. IgG-Sepharose where the IgG is specific against the heterologous polypeptide, is washed with a suitable medium and then incubated with the cleaving agent, such as protease or cyanogen bromide. After removal of the carrier material having the heterologous polypeptide bound thereto, a solution containing the cleaved desired polypeptide and the cleavage agent is obtained, from which the former may be isolated and optionally further purified by techniques known in the art such as gel filtration, ion-exchange etc.

Where the fusion polypeptide comprises a protease recognition site, the cleavage procedure may be performed in the following way. The affinity matrix-bound fusion polypeptide is washed with a suitable medium, and then

eluted with an appropriate agent which is as gentle as necessary to preserve the desired S3 multimer. Such an agent may, depending on the particular S3 multimer, be a pH-lowering agent such as a glycine buffer. The eluate containing the pure fusion polypeptide is then passed through a second column comprising the immobilized protease, e.g. collagenase when the cleavage site is a collagenase susceptible sequence. When passing therethrough the fusion polypeptide is cleaved into the desired S3 multimer and the heterologous polypeptide. The resulting solution is then passed through the same affinity matrix, or a different affinity matrix, to adsorb the heterologous polypeptide portion of the solution.

In one embodiment, we chose the amplification vector that readily allows us to obtain various multimers of S3 gene. Furthermore, we designed the Asp-Pro (DP) linker between the repetitive units, to afford convenient cleavage under mildly acidic buffer to release the monomers.

Studying the tandem repeats of S3 may provide explanations as to why some proteins adopt repetitive structure, and how they contribute strategically towards pathogen recognition. In one embodiment, tandem repeats of S3 gene were cloned into a modified vector, which was subsequently transferred to an expression vector, pET22b. Induced expression of the most robust tetramer clone was scaled-up. Recombinant S3 tetramer (rS3-4mer) was purified and digested into monomers (rS3-1mer) by acid treatment, and both the recombinant peptides were tested for their endotoxin binding and neutralizing activities. The rS3-1mer peptide has the additional D (asp) and P (pro) at the ends as a result of acid cleavage of the DP linker. Chemically synthesized S3 does not have these 2 extra amino acids.

The multimeric constructs exhibit different expression levels. No expression was observed with the pETS3-1mer. As the copy number increases, the expression level improved dramatically, especially with the S3 tetramer, where the expression level reached 25% of the total cell proteins. However, further doubling to 8mer reduced the expression level, suggesting that the copy number is not always proportional to the expression level for this peptide. The ELISA-based LPS binding test and SPR result show differential binding efficiencies of rS3-4mer, rS3-1mer and the chemically synthesized S3 for LPS, with highest binding achieved by rS3-4mer. Both the LAL inhibition test and suppression of TNF-α release in THP-1 cells showed that rS3-1mer works equally well as the chemically synthesized S3 peptide to neutralize LPS, while rS3-4mer displayed a 2-fold higher anti-LPS activity. However, the rS3-1mer and chemically synthesized S3 showed inconsistent results in ELISA and SPR tests.

Two major forces mediate the interaction between LPS and LPS-binding peptides. The positive charge on the peptides forms an electrostatic attraction with the negatively charged phosphate head groups of the LPS. The other is the hydrophobic interaction between them (Goh *et al*, 2002; Farley *et al*, 1988). In fact, mutation of amino acid residues of S3 aimed at introducing positive charges only achieved a slight increase in LPS-neutralizing activity (Tan *et al*, 2000b). Besides charge modification, little effort has been taken to enhance the LPS-binding ability of such peptides. Herein, by creating tandem repeats of the LPS-binding units instead of increasing the number of positive charges, we demonstrate a 2-fold improvement in activity of the tetramer compared to the original monomeric

unit, thus providing an alternative strategy to improve the LPS-binding activity of similar peptides.

The result of secondary structure analysis by DNAMAN program (Version 4.15, Lynnon Biosoft) shows that both S1 and S3 have a distinctive structure of four regular β-sheets alternately spaced by turns and coils. We presume that this structure may be important to the interaction with LPS, and in addition, the multiple β-sheets in rS3-4mer, may form the β-barrel structure to provide better shielding of hydrophobic acyl chain of LPS (Ferguson *et al*, 1998).

We have developed a specific endotoxin adsorption using rS3-4mer, which is a recombinant tetramer of S3. Its high binding affinity of LPS, lower cytotoxicity and haemolytic activity showed its advantage over the synthetic amphipathic cationic peptide, S3Δ (Ding *et al*, 2001). rS3-4mer was covalently conjugated to DADPA immobilized agarose beads. The efficacy of this matrix to remove LPS was tested under different conditions and compared with the S3Δ matrix.

Compared to S3Δ peptide, the rS3-4mer recombinant peptide appears to be on par if not better in terms of efficiency of LPS removal. The tandem repeats of the S3 might expose strategically positioned multiple LPS-binding motifs, which produce synergistic effect for binding LPS. Both of these peptides have great potential for the pyrogen clean-up industry, as they are re-usable, non-toxic and efficient in removing trace amounts of LPS from solutions. Better systems of removing LPS from solutions will be required to meet demands of new and tighter legislations.

In one aspect, the invention encompasses (i) an expression cassette containing a DNA molecule of the invention placed under the control of the elements required

for expression, in particular under the control of an appropriate promoter; (ii) an expression vector containing an expression cassette of the invention; (iii) a procaryotic or eucaryotic cell transformed or transfected with an expression cassette and/or vector of the invention, as well as (iv) a process for producing a polypeptide or polypeptide derivative encoded by a polynucleotide of the invention, which involves culturing a procaryotic or eucaryotic cell transformed or transfected with an expression cassette and/or vector of the invention, under conditions that allow expression of the DNA molecule of the invention and, recovering the encoded polypeptide or polypeptide derivative from the cell culture. It is understood that by the term "polypeptide" includes short amino acid sequences commonly termed "peptides".

A recombinant expression system is selected from procaryotic and eucaryotic hosts. Eucaryotic hosts include yeast cells (*e.g.*, *Saccharomyces cerevisiae* or *Pichia pastoris*), mammalian cells (*e.g.*, COS1, NIH3T3, or JEG3 cells), arthropods cells (*e.g.*, *Spodoptera frugiperda* (SF9) cells), and plant cells. A preferred expression system is a procaryotic host such as *E. coli*. Bacterial and eucaryotic cells are available from a number of different sources including commercial sources to those skilled in the art, *e.g.*, the American Type Culture Collection (ATCC; Rockville, Maryland). Commercial sources of cells used for recombinant protein expression also provide instructions for usage of the cells.

The choice of the expression system depends on the features desired for the expressed polypeptide. For example, it may be useful to produce a polypeptide of the invention in a particular lipidated form or any other form.

One skilled in the art would readily understand that not all vectors and expression control sequences and hosts would be expected to express equally well the polynucleotides of this invention. With the guidelines described below, however, a selection of vectors, expression control sequences and hosts may be made without undue experimentation and without departing from the scope of this invention.

In selecting a vector, the host must be chosen that is compatible with the vector which is to exist and possibly replicate in it. Considerations are made with respect to the vector copy number, the ability to control the copy number, expression of other proteins such as antibiotic resistance. In selecting an expression control sequence, a number of variables are considered. Among the important variable are the relative strength of the sequence (*e.g.* the ability to drive expression under various conditions), the ability to control the sequence's function, compatibility between the polynucleotide to be expressed and the control sequence (*e.g.* secondary structures are considered to avoid hairpin structures which prevent efficient transcription). In selecting the host, unicellular hosts are selected which are compatible with the selected vector, tolerant of any possible toxic effects of the expressed product, able to secrete the expressed product efficiently if such is desired, to be able to express the product in the desired conformation, to be easily scaled up, and to which ease of purification of the final product.

The choice of the expression cassette depends on the host system selected as well as the features desired for the expressed polypeptide. Typically, an expression cassette includes a promoter that is functional in the

selected host system and can be constitutive or inducible; a ribosome binding site; a start codon (ATG) if necessary; a region encoding a signal peptide, *e.g.*, a lipidation signal peptide; a DNA molecule of the invention; a stop codon; and optionally a 3' terminal region (translation and/or transcription terminator). The signal peptide encoding region is adjacent to the polynucleotide of the invention and placed in proper reading frame. The signal peptide-encoding region is homologous or heterologous to the DNA molecule encoding the mature polypeptide and is compatible with the secretion apparatus of the host used for expression. The open reading frame constituted by the DNA molecule of the invention, solely or together with the signal peptide, is placed under the control of the promoter so that transcription and translation occur in the host system. Promoters and signal peptide encoding regions are widely known and available to those skilled in the art and include, for example, the promoter of *Salmonella typhimurium* (and derivatives) that is inducible by arabinose (promoter araB) and is functional in Gram-negative bacteria such as *E. coli* (as described in U.S. Patent No. 5,028,530 and in Cagnon *et al.*, (Cagnon *et al.*, Protein Engineering (1991) 4(7):843)); the promoter of the gene of bacteriophage T7 encoding RNA polymerase, that is functional in a number of *E. coli* strains expressing T7 polymerase (described in U.S. Patent No. 4,952,496); OspA lipidation signal peptide ; and RlpB lipidation signal peptide (Takase *et al.*, J. Bact. (1987) 169:5692).

The expression cassette is typically part of an expression vector, which is selected for its ability to replicate in the chosen expression system. Expression vectors (*e.g.*, plasmids or viral vectors) can be chosen, for example, from those described in Pouwels *et al.* (Cloning

Vectors: A Laboratory Manual 1985, Supp. 1987). Suitable expression vectors can be purchased from various commercial sources.

Methods for transforming/transfecting host cells with expression vectors are well-known in the art and depend on the host system selected as described in Ausubel *et al.*, (Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons Inc., 1994).

Upon expression, a recombinant polypeptide of the invention (or a polypeptide derivative) is produced and remains in the intracellular compartment, is secreted/ excreted in the extracellular medium or in the periplasmic space, or is embedded in the cellular membrane. The polypeptide is recovered in a substantially purified form from the cell extract or from the supernatant after centrifugation of the recombinant cell culture. The recombinant polypeptide is purified by any well-known methods that can be readily adapted by a person skilled in the art, such as fusion of the polynucleotide encoding the polypeptide or its derivative to a small affinity binding domain.

In various embodiments as described above, a polypeptide may be cleaved to obtain the desired multimer or monomer. Proteolytic cleavage can be done by methods known in the art. One method may be acid digestion. Alternatively, a proteolytic cleavage site may be introduced at the junctions so that the desired peptide can ultimately be separated from the remainder of the polypeptide. Proteolytic enzymes include, but are not limited to, factor Xa, thrombin, and enterokinase.

By "S3 multimer" is meant a polypeptide having more than one copy of S3 peptide, including multiple tandem repeats of S3 peptide. A DNA sequence encoding S3 multimer contains the S3 peptide-encoding sequence within a single open reading frame such that when expressed, S3 multimer is produced. The S3 peptide sequences may all be the same, or may correspond to different derivatives, analogs, variants such as allelic variants, and homologs of S3 peptide so long as they retain the ability to bind to LPS. Examples of S3 peptide include SEQ ID NOs: 1 and 2. An exemplary cDNA encoding Factor C of *Carcinoscorpius rotundicauda* is set forth in SEQ ID NOs: 5 and 6. SEQ ID NO:1 corresponds to amino acids 268-301 of SEQ ID NO:6.

If the S3 peptides are linked either chemically or recombinantly to a heterologous polypeptide, they may be linked to either the 5'-end, the 3'-end, or may flank the heterologous polypeptide. Further, the S3 multimer may be located at sites internal to the heterologous polypeptide.

In certain embodiments, S3 multimer may contain 2, 3, 4, 5, 6, 7, 8, 9 or 10 S3 peptide sequences. The S3 peptides may be present as direct tamdem repeats, or they may be separated by linking amino acids or cleavage sites at junctions between some of the repeats.

By "polypeptide" or "protein" is meant any chain of amino acids, regardless of length or post-translational modification (*e.g.*, glycosylation or phosphorylation), or chemical modification, or those containing unnatural or unusual amino acids such as D-Tyr, ornithine, amino-adipic acid. Both terms are used interchangeably in the present application.

Spacer sequences may be present between the S3 moieties. The strategic placement of various spacer sequences between S3 sequences can be used to confer even greater LPS-binding. Accordingly, a selected spacer sequence may encode a wide variety of moieties such as a single amino acid linker or a sequence of two to several amino acids. Selected spacer groups may preferably provide enzyme cleavage sites so that the expressed multimer can be processed by proteolytic enzymes in vivo (by APCs, or the like).

In various embodiments, a peptide may be labeled. The peptides may be labeled at any position in the amino acid sequence, such as at the N-termini, C-termini, or at an amino acid side chain (e.g., Lys, Arg, Ser, Cys, Tyr, Glu, Asp, etc.). However, since not all side chains will be present in all of the peptides produced in the digest, labeling at the N-, or C-termini is preferred. N-terminal peptide labeling is particularly preferred.

Preferred labeling groups are fluorescent chromophores that are conventionally used as reporter groups. For example, the structurally related cyanine (Cy.TM.) fluorescent labeling reagents, Cy3 and Cy5, may be used to produce N-terminally-tagged peptides. Incubation of the Cy3 or Cy5 monofunctional succinimide esters with the peptide will result in N-terminal labeling of the peptides. These dyes are commercially available from Amersham Pharmacia Biotech.

Alexa™ dyes marketed by Molecular Probes, Inc. may also be used. These dyes comprise a series of fluorophores with emission maxima throughout the visible spectrum. Of these, two dyes, Alexa 532 and Alexa 568 would be especially suitable. Both share a similar fluorophore and bear the same

polar sulfonate and quaternary nitrogen functional groups in similar spatial orientation in the molecule. Their emission maxima are at 554 nm and 603 nm, respectively.

A variety of techniques well-known for separating peptides may be used to separate and detect the peptides and their multimers. For example, such techniques include 2D gel electrophoresis, capillary electrophoresis, isoelectric focusing and liquid chromatography, and high-performance liquid chromatography (HPLC). Reverse-phase HPLC is a routine analytical procedure in the field of protein and peptide analysis.

In reverse phase HPLC C-18 columns typically are used, although shorter-chain stationary phases provide improved resolution for larger polypeptides. Three column formats are most widely used. Analytical columns (4.6 mm I.D.) typically are eluted at flow rates of 0.5-2 mL min^{-1}. Narrow bore columns (1 mm I.D.) are run at approximately 0.1 mL min^{-1}. Fused silica capillary columns (0.1-0.3 mm I.D.) are eluted a flow rates of 4μL min^{-1} and below.

In various embodiments, the polypeptides of the invention may be immobilized on solid phase media. Generally, the matrix provides a scaffold which allows the polypeptide to which it is linked to be separated from the bulk fluid. Non-limiting examples of suitable matrices include: polymers which are insoluble in water or mixtures of water and water soluble organic solvents; beaded supports such as magnetic beads, chromatographic packing supports, media and resins; porous beaded supports such as chromatographic packing supports, media and resins (e.g. anion exchange chromatography media), a cast polymer such as a membrane (e.g. polyvinylidene difluoride, Teflon,

polyethylene, polypropylene or polysulfone); co-polymeric materials and gels (e.g. polyacrylamide or agarose).

In various embodiments, the polypeptides of the invention may be used therapeutically in formulations or medicaments to prevent or treat sepsis-related disease. The invention provides corresponding methods of medical treatment, in which a therapeutic dose is administered in a pharmacologically acceptable formulation, e.g. to a patient or subject in need thereof. Accordingly, the invention also provides therapeutic compositions comprising the polypeptide of the invention, and a pharmacologically acceptable excipient or carrier. In one embodiment, such compositions include the polypeptide of the invention in a therapeutically or prophylactically effective amount sufficient to treat sepsis-related conditions. The therapeutic composition may be soluble in an aqueous solution at a physiologically acceptable pH.

A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result, such as a reduction of symptoms related to sepsis and in turn a reduction in sepsis-related disease progression. A therapeutically effective amount of the polypeptide of the invention may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the compound to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. A therapeutically effective amount is also one in which any toxic or detrimental effects of the compound are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of

time necessary, to achieve the desired prophylactic result, such as preventing or inhibiting the rate of sepsis onset or progression. A prophylactically effective amount can be determined as described above for the therapeutically effective amount. For any particular subject, specific dosage regimens may be adjusted over time according to the individual need and the professional judgement of the person administering or supervising the administration of the compositions.

As used herein "pharmaceutically acceptable carrier" or "excipient" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. In one embodiment, the carrier is suitable for parenteral administration. Alternatively, the carrier can be suitable for intravenous, intraperitoneal, intramuscular, sublingual or oral administration. Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or

dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, monostearate salts and gelatin. Moreover, the polypeptide of the invention can be administered in a time release formulation, for example in a composition which includes a slow release polymer. The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, polylactic acid and polylactic, polyglycolic copolymers (PLG). Many methods for the preparation of such formulations are patented or generally known to those skilled in the art.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g. the polypeptide of the invention) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients

from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. In accordance with an alternative aspect of the invention, the polypeptide of the invention may be formulated with one or more additional compounds that enhance the solubility of the polypeptide.

In accordance with another aspect of the invention, therapeutic compositions of the present invention, comprising the polypeptide of the invention, may be provided in containers or commercial packages which further comprise instructions for use of the polypeptide of the invention, in the prevention and/or treatment of sepsis-related disease, or for the preparation of a medicament for prevention and/or treatment of sepsis-related disease.

The following preparative and biological examples are offered to illustrate this invention and are not to be construed in any way as limiting the scope of this invention.

EXAMPLES

LPS from *Escherichia coli* 055:B5 was purchased from Sigma (St. Louis, MO.). LAL kinetic-QCL kit was supplied by BioWhittaker (Walkersvile, MD). Human TNF-α kit (OptEIA ELISA) was from Pharmingen (San Diego, CA). CellTiter 96 Aqueous One Solution Reagent for cytotoxicity assay was purchased from Promega (Madison, WI). Enzymes for DNA manipulation and polymerase reactions were purchased from NEB (Beverly, MA.). DNA purification and extraction kits were from Qiagen (Chatsworth, CA). Pyrogen-free water

for making buffers was from Baxter (Morton Grove, IL). Chemically synthesized S3 peptides were made commercially according to sequence information supplied by us.

rS3-4mer is a tetramer with tandem repeat of the S3 gene. An endotoxin removing affinity matrix was developed by using 1-ethyl-3- (3- dimethylaminopropyl) carbodiimide (EDC) as a coupling agent to link rS3-4mer to DADPA-immobilized agarose gel (Pierce, USA). The rationale for choosing DADPA-immobilized agarose matrix is based on reference (Ding et al. 2001). The LAL Kinetic-QCL assay kit was from BioWhittaker, USA. LPS (*Escherichia coli* 055:B5) and sodium deoxycholate (DOC) were from Sigma. RPMI 1640 was from Gibco, BRL. Chymotrypsinogen A was purchased from Pharmacia Biotech. All other chemicals were of analytical grade from Sigma.

All materials relating to the affinity matrix studies were depyrogenated. Glassware and sodium chloride were baked at 200°C for 4 hours. Sterile disposables were used whenever possible and were autoclaved at 121°C for 2 hours before use. All solutions and buffers were prepared using pyrogen-free water. LPS was sonicated for 5 minutes in a 37°C water bath prior to use, to disperse the aggregates.

(1) *Construction of multimers of S3 gene*

Using a cloned Factor C Sushi3 domain, pAC5.1Sushi3EGFP (Tan *et al*, 2000), the LPS-binding motif, S3, was amplified by PCR. A cloning strategy, which allows for directional multimerization and cloning is shown in Figure 1. Briefly, the amplification vector pBBSI (Lee *et al*, 1997) was modified to include an *Nde*I site containing the start codon adjacent to *BbsI* site. This modified vector

was named pBC. Forward primer (SEQ ID NO:3): 5'-TCGAAGAC*GGCCCC*AGGATCCCCATGCTGAACACAAGG-3' was designed with *Bbs*I restriction site (GAAGAC) followed by *GGCCCC* in addition to the S3 flanking sequence. On the reverse primer (SEQ ID NO:4): 5'TAGAAGAC*CCGGGGG*TCCATCAAAGAAAGTAGTTA-3', similar motif was also introduced. Digestion of the PCR product by *Bbs*I would yield fragments with complementary overhang of *CCCC* on the sense strand and *GGGG* on the antisense strand, which can be used for directional multimerization and cloning. In addition, GATCCC sequence, which codes for aspartate (D) and proline (P), was introduced into the forward primer. The peptide bond between D and P can be cleaved under acidic condition (Szoka *et al*, 1986), thus releasing single S3 units from the recombinant multimers. In this case, the PCR products of S3 were cloned into pBC vector, and the S3 gene was released by *Bbs*I digestion and allowed to self-ligate first, before cloning into the pBC vector, which was previously linearised with *Bbs*I. The multimers of S3 gene were selected and identified by enzyme digestion and sequencing.

(2) *Expression of the multimers of S3 gene in E. coli*

To construct expression vectors bearing tandem S3 genes under the control of T7 promoter, the fragments flanked by *Nde*I and *Hind*III (containing the multimeric S3 genes) were cloned into the vector pET22b, previously linearised with *Nde*I and *Hind*III. The constructs were transformed into *E. coli* host, BL21 (DE3) for expression. The colonies were cultured overnight in LB medium with 100 µg /ml ampicillin at 37°C, then diluted 1:100 into fresh LB medium with 100 µg/ml ampicillin and grown to OD_{600nm} of 0.6 before induction with 0.5 mM IPTG (Promega). The cells were

harvested every h up to 12 h, and the expressed products were monitored by SDS-PAGE.

(3) *Solubilization of inclusion bodies and purification of rS3-4mer*

One litre cultures were pelleted at 5000g for 10 min at 4°C and resuspended in 60 ml of lysis buffer containing 20 mM Tris-Cl, pH 8.0 and 0.5 mM DTT. The bacterial cells in the suspension were passed through French Press (Basic Z 0.75KW Benchtop Cell Disruptor, UK) operated at 15 kpsi, for 4 rounds in order to generate > 90% cell disruption. The inclusion bodies were recovered by centrifugation at 12000g for 20 min at 4°C and washed with 20 mM Tris-Cl buffer containing 1 M urea and 0.5% Triton X-100. The inclusion bodies were denatured and solubilized in 20 mM Tris-Cl with 8 M urea at room temperature for 2 h. Insoluble materials were removed by centrifugation at 16000g for 20 min, and the supernatant was filtered and purified by anion exchange using ÄKTA explorer (Pharmacia). Briefly, 30 ml of solubilized proteins were applied to a Q-Sepharose column (26 mm x 300 mm) equilibrated with buffer A (4 M urea, 20 mM Tris-Cl, pH 6.7). After washing with 4 column volumes of buffer A, the proteins were eluted with a linear gradient of 0% to 30% buffer B (4 M urea, 20 mM Tris-Cl, pH6.7, 1 M NaCl) and the fractions were collected for SDS-PAGE analysis. The collected fractions were pooled and dialyzed in 10 kDa molecular weight cut-off (MWCO) pore size dialysis tubing (Snakeskin, Pierce), against refolding buffer A containing 50 mM glycine, pH 9.5, 10% sucrose, 1 mM EDTA, 2 M urea, at 4°C for 16 h, followed by buffer B containing 20 mM diethanolamine, pH 9.5, 10% (w/v) sucrose, 1 mM EDTA, 4°C for another 8 h.

(4) *Monomerization of rS3-4mer (SEQ ID NO:9) into rS3-1mer (SEQ ID NO:7) by acid digestion*

Two adjacent amino acids, aspartate and proline were added between the S3 units, so as to act as cleavable DP linkers. The renatured rS3-4mer was precipitated with 9 volumes of ethanol, frozen at -80°C for 1 h or at -20°C overnight. The mixture was centrifuged at 16000g for 10 min and the pellet was washed with 90% ethanol, dried, dissolved in digestion buffer (70% formic acid, 6 M guanidine-Cl) and digested at 42°C for 72 h. The final products were subjected to ethanol precipitation and dissolved in 20 mM Tris-Cl pH 7.3. The cleaved rS3 peptides were then dialyzed overnight against the same buffer using dialysis tubing of 1.5 kDa MWCO pore size (Sigma), thus removing the small linkers and residual salt. The endotoxin contaminant in rS3-4mer and rS3-1mer was removed by Triton X-114 phase separation (Liu *et al*, 1997) followed by polymyxin B affinity chromatography (Detoxi-Gel™, Pierce).

Tricine SDS-PAGE and Western blot analysis: The recombinant proteins were resolved on tricine SDS-PAGE, using 5% stacking gel and 15% separating gel, and detected by Coomassie blue staining (Schagger *et al*, 1987). Western analysis was performed according to the manufacturer's instruction, using ECL Western analysis system (Pierce, IL). The blot was probed with polyclonal rabbit anti-S3 antibody followed by goat anti-rabbit secondary antibody conjugated to horseradish peroxidase, HRP (DAKO, CA). The blots were visualised using Supersignal West Pico Chemiluminescent Substrate and exposed to X-ray film.

(5) *Assays for LPS-neutralizing activity*

(a) ELISA-based LPS binding assay

The polysorp 96-well plate (MaxiSorpTM, Nunc) was first coated with 100 µl per well of 4 µg/ml (approximately 1 µM) of LPS diluted in pyrogen-free phosphate-buffered saline (PBS). The plate was sealed and incubated overnight at room temperature. The wells were aspirated and washed 4 times with 300 µl wash solution (PBS containing 0.05% Tween-20). The wells were blocked with wash solution containing 2% BSA for 1 h at room temperature. After washing 2 times, varying concentrations of peptides were allowed to interact with bound LPS at room temperature for 3 h. Bound peptides were detected by incubation with rabbit anti-S3 antibody and 1:2000 of goat anti-rabbit antibody conjugated with HRP. Each antibody was incubated for 2 h at 37°C. In the final step, 100 µl of substrate, ABTS (Boehringer Mannheim), was added. The absorbance was measured at 405nm with reference wavelength at 490nm.

(b) Endotoxin neutralization assay based on anti-LAL test

The LAL Kinetic-QCL kit utilizes the initial part of the LPS-triggered cascade in limulus amoebocyte lysate to achieve an enzymatic reaction, which catalyses the release of p-nitroaniline from a synthetic substrate, producing a yellow color, which is quantifiable by absorbance at 405_{nm}. The ENC_{50} (Endotoxin Neutralization Concentration) refers to the peptide concentration required to neutralize 50% of a predetermined quantity of endotoxin. A low ENC_{50} indicates high potency of the peptide for endotoxin neutralization.

In this assay, peptides of different concentrations were incubated for 1 h at 37°C with or

without an equal volume of LPS in disposable, endotoxin-free borosilicate tubes. Fifty microliters of each mixture was then dispensed into wells of a sterile microtiter plate (Nunclon™ Δsurface, Nunc). Fifty microliters of freshly reconstituted LAL reagent was dispensed into each well. The absorbance at 405_{nm} of each well was monitored after 45 min, and the concentration of peptides corresponding to 50% inhibition of LAL activity was designated ENC_{50}.

(6) *Suppression of LPS-induced hTNF-α secretion in human THP-1 cells*

THP-1 cells were cultured at 37°C in a humidified environment in the presence of 5% CO_2. RPMI 1640 medium was supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 µg/ml). The cells were maintained at a density of $2.5x10^{5-6}$ cells/ml. THP-1 monocytes were transformed into macrophages by addition of phorbol myristic acid, PMA (Sigma) at a stock of 0.3 mg/ml in dimethyl sulfoxide to give a final concentration of 30 ng/ml and 0.01% dimethyl sulfoxide. PMA-treated cell suspensions were immediately plated into 96-well microtiter plate at a density of $4x10^5$ cells/ml and allowed to differentiate for 48 h at 37°C. The culture medium was removed and the cells were washed twice with serum-free RPMI 1640. Thereafter, the macrophages were stimulated with 50 EU/ml LPS (a specific activity of LPS that has been standardized by LAL test against FDA-approved LPS standards), peptides alone or LPS (preincubated with various concentrations of peptides) and incubated at 37°C. After 6 h, the culture medium was collected and hTNF-α concentration in the supernatants was assayed using ELISA.

(7) *Realtime interaction analysis between peptides and LPS*

Surface plasmon resonance (SPR) analysis of the real time interaction between peptides and LPS was performed with BIAcore 2000 (Pharmacia) using HPA chip (Tan *et al*, 2000b). The affinity constant was calculated using BIAevaluation software 3.0. The mean values were obtained from three independent experiments.

(8) *Cytotoxicity of peptides in eukaryotic cells*

THP-1 monocytes in 50 μl of 2×10^4 cells/ml in RPMI 1640 were mixed in a microtiter plate with 50 μl of two-fold serial dilutions of peptides ranging in concentration, and incubated for 60 min at 37°C. To determine the cytotoxicity induced by the peptides, 20 µl of CellTiter 96 Aqueous One Solution Reagent was added into each well for 90 min at 37°C. MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] is bioreduced by metabolically active cells into a colored formazan product that is soluble in tissue culture medium. For detection, the absorbance was measured at 490_{nm}. To determine the ratio of cell lysis induced by the peptides, two controls were included by incubating cells in PBS containing 0.2% Tween-20 instead of medium only. This absorbance value corresponds to the background, as those cells could not metabolize MTS.

(9) Data Analysis of S3 Tandem Repeats Studies

Recombinant expression of S3 tandem repeats, purification and cleavage to monomers

A 143 bp S3 gene fragment was obtained by PCR using pAC5.1Sushi3EGFP as the template. The S3 gene was cloned into pBC vector by digestion with *Bbs*I. After

multimerization, the clones containing 1, 2, 4 and 8 copies of S3 were selected (Figure 2a) and named pBCS3-1, -2, -4, -8mer, respectively. The *Nde*I and *Hind*III-flanking fragments of these clones were inserted into pET22b for expression of the multimeric S3 gene, and the expression levels were examined by SDS-PAGE. An exemplary sequence encoding rS3-4mer is shown in SEQ ID NO:8.

Of all the expression cassettes, the tetramer yielded the highest expression level, giving the expected recombinant S3 tetramer (rS3-4mer) of 18.4 kDa, which represented 25% of the total cell proteins (Figure 2b). The monomer construct was not expression-competent, while the octamer construct expressed poorly.

The rS3-4mer was expressed as inclusion bodies in *E. coli*. The solubilization in 8 M urea and purification through Q-Sepharose anion exchange chromatography produced more than 95% purity of rS3-4mer (Figure 2b), yielding 42 mg rS3-4mer per litre of culture. The purified protein was dialyzed and urea was removed gradually to allow the samples to refold. Dialysis also removed unspecific small molecular weight bacteria proteins, hence further improving the purity of the rS3-4mer. SDS-PAGE under non-reducing conditions showed majority of one band with the expected size (data not shown). A minor form of a larger aggregate was removed by size exclusion using Superose® 12 column (Pharmacia). The refolded protein was precipitated with 90% ethanol and redissolved in acid digestion buffer to obtain the monomers (rS3-1mer). The process of acid digestion is time dependent. A one-day treatment yielded polymeric mixtures of four kinds of rS3 peptides: rS3-4, -3, -2, -1mer (Figure 3a). Within 2 days, more than 90% of the multimers was cleaved to the monomers.

Recombinant Sushi3 peptides show stronger binding potency to LPS

Samples from the total cell proteins, purified rS3-4mer, partially digested rS3 polymers, rS3-1mer and chemically synthesized S3 peptide were resolved on tricine SDS-PAGE and subjected to Western analysis against anti-S3 antibody. The rS3-1mer and its partially digested polymeric repeats (2, 3 and 4mers) were immunoreactive to the polyclonal rabbit anti-S3 antibody (Figure 3b). Thus, the antibody can be employed for the ELISA-based LPS binding assay.

ELISA-based LPS binding assay revealed different binding capabilities with rS3-1 mer, -4mer and chemically synthesized S3. At 4 μg/ml, both recombinant peptides reached saturation of binding to LPS (Figure 4), while the chemically synthesized peptide continued linearly and required 20 μg/ml to reach saturation of binding with LPS (data not shown). The EBC_{50} (Endotoxin Binding Concentration) of the peptide, which achieves 50% of maximum binding to LPS on the ELISA plate, reflects the binding activity of peptide to LPS, with the lower EBC_{50} indicating higher potency. The rS3-4mer, rS3-1mer and chemically synthesized S3 peptides displayed EBC_{50} at 0.41 μg/ml, 1.02 μg/ml and 9.74 μg/ml, respectively. The kinetics of binding of peptides to LPS in 20 mM Tris-Cl, pH7.3, was also measured by SPR analysis with BIAcore 2000 using HPA chip, which was immobilized with lipid A (bioactive moiety of LPS). The *Kd* values of chemically synthesized S3, rS3-1mer and rS3-4mer are $(7.80\pm2.18) \times10^{-7}$ M, $(4.74\pm2.34) \times10^{-8}$ M, $(1.71\pm1.86) \times10^{-8}$ M, respectively.

The recombinant S3 peptides inhibit endotoxin-induced LAL reaction and hTNF-α release from THP-1 cells

The 50% endotoxin-neutralizing concentration (ENC_{50}) value of the peptides against 5 EU/ml of LPS was determined to be 5.4 µg/ml for rS3-4mer, 9.2 µg/ml for rS3-1mer, and 10.2 µg/ml for chemically synthesized S3 (Figure 5a). A lower ENC_{50} indicates higher potency of endotoxin neutralization. The binding isotherm of the two monomeric peptides, whether it is recombinant or synthetic is similar, but rS3-4mer shows a 2-fold stronger LPS neutralization efficacy.

Similar results were also obtained by measuring the ability of rS3-4mer & -1mer to inhibit LPS-induced hTNF-α production by THP-1 cells, which were incubated with 50 EU/ml of LPS containing various concentrations of peptides. As shown in Figure 5b, rS3-1mer required 83.2 µg/ml, whereas rS3-4mer required 40.4 µg/ml to achieve 50% inhibition.

The peptides show minimal cytotoxicity to eukaryotic cells

Both recombinant peptides had minimal effect on cell permeabilization. At the highest concentration of 50 µM, only 2-3% of cell lysis was caused by rS3-4mer, indicating that the recombinant multimers of S3 would have negligible contraindications, although the LPS binding activity is amplified significantly.

(10) *Recombinant sushi 3 peptides (rS3) labeled with quantum dot (QD) tags bacteria*

rS3 purified from recombinant bacteria, was labeled with QD (referred to as QD-rS3) was used as a novel fluorescent tag to detect the presence of bacteria. As an example to demonstrate the concept of specific tagging of

certain bacteria by QD-rS3, experiments were carried out with 2 strains for gram negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and a gram positive bacteria, *Staphylococcus aureus*). By addition of small quantities of QD-rS3 to overnight cultures of the bacteria, one observes, within minutes, the fluorescently labeled gram negative bacteria (which contain lipopolysaccharide, LPS to which rS3 specifically bind to) and no labeling of *S. aureus*. QD labeled rS3 is stable and emits strong fluorescence which readily biosenses the presence of gram negative bacteria.

This method can be applied to tagging bacteria in solution and in solid phase.

(11) *Covalent conjugation of rS3-4mer to DADPA-immobilized agarose beads*

rS3-4mer affinity matrix was prepared using DADPA-immobilized agarose. The peptide was dissolved in 1ml conjugation buffer [0.1M 2-(N-morpholino)ethanesulfonic acid (MES), pH 4.7] and used for conjugation by EDC, to DADPA-agarose packed in a column. After 3 hours of incubation, the column was drained and washed with water. These eluents were collected in 1 ml fractions. The amount of peptide immobilized to the matrix was determined by measuring the absorbance of the fractions at 280nm.

Beads were regenerated with 10-column volumes of 1% DOC, followed by 10-column volumes of pyrogen-free water, 2 M NaCl and pyrogen-free water. When not in use, the peptide-conjugated beads were stored at 4°C with 0.02% sodium azide to prevent the growth of bacteria.

(12) *LPS adsorption assay*

After regeneration of the column and equilibration in the appropriate buffer, the LPS-binding capacity of rS3-4mer-coupled beads was tested batchwise. An aliquot of 5 ml of standard LPS solution (under different pH and in the presence or absence of chymotrypsinogen A and EDTA) was allowed to flow through the column twice at room temperature. Solutions before and after the treatment were collected to determine the LPS removal and protein recovery. EDTA was added in order to reduce the affinity of LPS to proteins. This helps to improve the removal of low levels of LPS from protein solutions. (Petsch 2000).

(13) *Quantification of LPS and proteins*

LPS was quantified by using the LAL chromogenic kinetic assay. The solutions were sonicated and diluted in borosilicate glass tubes at 37°C. Fifty µl of each mixture was carefully dispensed into appropriate wells of a sterile 96-well microtitre plate (Nunclon™ Δ surface, Nunc). The reaction mixture was then incubated for 15 minutes at 37°C before 50µl of LAL reagent was added. The reaction mixtures were read at 405 nm every 5 minutes with a SPECTRAmax 340 plate reader running on SOFTmax PRO version 1.2.0. The temperature was kept at 37 °C during the incubation and measuring. Five LPS standards ranging from 0.005EU/ml to 50EU/ml were used to calibrate the absorbance into endotoxin concentration in EU/ml. A blank using pyrogen-free water and negative controls of only the peptides in buffer solution were also set up in the assay. All samples, blank, negative control and standards were in triplicates. Pyrogen-free water was used for dilution of reagents and peptides.

The protein concentration in sample solutions of before and after treatment was determined by measuring the absorbance at 280nm with a spectrophotometer DU 650 (Beckman).

(14) *Data analysis of LPS affinity studies*

LPS affinity matrix preparation

In preparing the rS3-4mer conjugated affinity matrix for LPS, 1.5 mg/ml of peptides was used for coupling. 1.27mg/ml of peptides was successfully coupled onto the affinity matrix with coupling efficiency of 87% achieved, based on the following calculations:

A_{280nm} before coupling (abs) = 1.62

A_{280nm} of fraction recovery (abs) = 0.0601 + 0.316 + 0.0472 + 0.0204 + 0.0099 + 0.0033 + 0.0016 = 0.21

Total protein coupled on (abs) = 1.62 - 0.21 = 1.41

Coupling efficiency = 1.41 / 1.62 = 87%

LPS binding by rS3-4mer affinity beads at various endotoxin concentrations

It is reported that the usual LPS problematic contamination level is up to 100EU/ml after the initial steps of purification. Thus, we tested the DADPA rS3-4mer and S3Δ column at these endotoxin concentrations. When buffer solutions containing 20mM Tris-HCl, 100mM NaCl and 0.5M EDTA of pH7.3 were used, both spiked levels of 5 and 500 EU/ml were reduced to below the detection limit of 0.005EU/ml for both the columns.

However, LPS is normally found together with proteins such as in pharmaceutical fluids. Therefore, there is a need to test the protein recovery and LPS removal under such conditions. There are electrostatic and hydrophilic interactions between proteins and LPS. These interactions may reduce the efficiency of LPS removal. Very often, high protein recovery will compromise the removal of LPS from the solution and vice versa. In this work, 0.5mg/ml of chymotrypsinogen A was spiked into the same Tris buffer and was passed through the column twice to test the ability of the conjugated agarose beads for selective removal of LPS from protein solutions.

For both the columns, the LPS-affinity beads were able to remove LPS to a concentration below the detection limit of 0.005EU/ml. However, the rS3-4mer column appears to be able to recover more proteins compared to the S3Δ column under the current process conditions (Table 1).

Table 1

Removal of LPS from various solutions with problematic low levels of LPS contamination[a]

Column	DADPA rS3\4mer				
	Tris buffer[b] (+EDTA)[c]			Chym. A[b] (+EDTA)[c]	
	LPS (EU/ml)				Protein recovery[e] (mg/ml)
Before treatment	5	50	100	50	0.5
After treatment	<0.005[d]	<0.005[d]	<0.005[d]	<0.005[d]	0.4175
Clearance factor (CF)	>1000	>10000	>20000	>10000	83.5% recovered

Column	DADPA S3Δ		
	Tris buffer[b] (+EDTA)[c]	Chym. A[b] (+EDTA)[c]	
	LPS (EU/ml)		Protein recovery[e] (mg/ml)
Before treatment	5	100	0.5
After treatment	<0.005[d]	<0.005[d]	0.3125
Clearance factor (CF)	>1000	>20000	62.5% recovered

[a] Aliquots of 5 ml of each sample solution containing LPS at indicated concentrations were passed through a column with 1ml of affinity beads. LPS concentration of samples was measured by LAL chromogenic assay.

[b] Tris buffer: 20 mM Tris-HCl, pH 7.3 containing 100mM NaCl; Chym. A: 0.5 mg/ml chymotrypsinogen A in same Tris buffer

[c] EDTA at stock solution of 0.5 M (pH7.3) was added to the buffer to achieve a final concentration of 5mM before running through column.

[d] 0.005EU/ml is the detection limit of LAL chromogenic assay.

[e] A_{280nm} intensity of the samples before and after treatment with beads were measured to calculate the protein recovery.

Characteristics of LPS binding by rS3-4mer affinity beads

To determine if the peptide is able to remove LPS under a wide range of pH, buffer solutions of different pH containing 100mM of NaCl were tested. Increase in pH was expected to cause electrostatic interaction between the

negatively charged phosphate groups of LPS and the positively charged peptides to weaken and decrease the binding capacity of the peptide (Ding et al. 2001).

We observe a significant drop in efficiency of LPS removal when the pH shifted from 5.0 to 6.0. This could be because pH of 6.0 is very near the isoelectric point of the rS3-4mer, peptide, thus causing the electrostatic interactions to weaken and hence, it loses its binding capacity for LPS. When the pH shifted from 6.0 to 7.3, the efficiency of these affinity beads was enhanced with clearance factor of more than 450. Further increase in pH causes the clearance factor to drop drastically to only 4.3, this maybe due to the increase of negative charge on peptide, so decrease the affinity of peptide to LPS, since the LPS is strongly negatively charged.

From the results, the rS3-4mer is able to remove LPS under a relatively wide range of pH, but with efficiencies highest only at around pH of 7.3 (Table 2).

Table 2

Removal of LPS from various solutions with problematic low levels of LPS contamination[a]

Column	DADPA rS3\4mer				DADPA S3Δ
	Sodium acetate buffer[b]		Tris buffer[c]		
pH[d]	5.0	6.0	7.3	8.5	7.3
Before treatment (EU/ml)	500				
After treatment (EU/ml)	4.616	61.38	1.090	116.84	3.176
Clearance factor (CF)	108	8	459	4.3	157

[a] Aliquots of 5 ml of each sample solution containing LPS at indicated concentrations were passed through a column with 1ml of affinity beads. LPS concentration of samples was measured by LAL chromogenic assay.

[b] Sodium acetate buffer: 20 mM sodium acetate containing 100mM NaCl;

[c] Tris buffer: 20 mM Tris-HCl containing 100mM NaCl;

[d] pH adjusted to around 7 for LAL test by adding 5% of 1M Tris-HCl, pH7.3 for acidic samples and 4% 1M NaAc, pH5.0 for the pH8.5 sample.

[e] Clearance Factor (CF) which reflects the efficiency of LPS removal, is obtained by dividing concentration of LPS before with after treatment.

Removal of LPS in tissue culture medium

For the cell culture medium RPMI 1640, both columns were able to remove LPS from 120 EU/ml to below the detection limit of 0.005 EU/ml.

Table 3

Removal of LPS from culture medium with problematic low level of LPS contamination[a]

	Medium[b]	
Column	DADPA rS3\4mer	DADPA S3Δ
Before Treatment (EU/ml)	120	120
After treatment (EU/ml)	<0.005[c]	<0.005[c]
Clearance Factor (CF)	>24000	>24000

[a] Aliquots of 5 ml of each sample solution containing LPS at indicated concentrations were passed through a column with 1ml of affinity beads. LPS concentration of samples was measured by LAL chromogenic assay.

[b] Medium: RPMI 1640 pH 9.0

[c] 0.005EU/ml is the detection limit of LAL chromogenic assay.

REFERENCES

de Haas, C.J.C., M.E.van der Tol., K.P.M. Van Kessel, J. Verhoef and J.A.G. Van Strijp.(1998) J. Immumol. 161:3607-3615.

Ding, J.L., Navas, M.A.A. and Ho, B.(1995) Mol. Marine Biol. Biotech., 4:90-103.

Ding, J.L., Zhu, Y., and Ho, B. (2001) J. Chromatography., 795:237-246.

Dolby, N., Dombrowski, K.E. and Wright, S.E. (1999) Protein Expres Purif.15:146-154.

Farley, M.M., Shafer, W.M. and Spitznagel, J.K. (1988) Infect Immun. 56:1589-1592.

Ferguson, A.D., Hofmann, E., Coulton, J.W., Diederiches, K. and Welte, W. (1998) Science. 5397: 2215-2220.

Goh, Y.Y., Frecer, V., Ho, B. and Ding, J.L. (2002) Protein Eng. 15:493-502.

Ho, B. (1983) Microbios Lett. 24: 81-84.

Kajino,T., Takahashi, H., Hirai, M. and Yamada, Y. (2000) Appl Environ Microbiol. 66:304-309.

Latham, P.W. (1999) Nature Biotech. 17:755-757.

Le, H.V. and Trotta, P.P. (1991) Bioprocess Technol.12:163-81.

Lee, J.H., Minn, I., Park, C.B. and Kim, S.C. (1998) Protein Expres Purif. 12:53-60.

Lee, S.J., Lee, J.H., Jin, H.J., Ryu, H.Y., Kim, Y., Kong, S. and Kim, K.W. (2000) Mol Cells. 10:236-240.

Liu, S.G., Tobias, R., McClure, S., Styba, G., Shi, Q.W. and Jackowski, G. (1997) Clin Biochem. 6:455-463.

Mauro, J.M. and Pazirandeh, M. (2000) Lett Appl Microbiol. 2:161-166.

S. Minobe, S., Watanabe, T., Sata, T., Tosa, T., Chibata, J. Preparation of adsorbents for pyrogen adsorption. J. Chromatography. 248 (1982) 401.

Petsch, D., Anspac, F.B. Endotoxin removal from protein solutions. J. Biotechnology. 76 (2000) 97.

Schagger, H. and von Jagow, G. (1987). Anal Biochem. 166: 368-379.

Scott, M.G., Vreugdenhil, A.C.E., Buurman, W.A., Hancock, R.E.W. and Gold, M.R. (2000) J Immunol., 164: 549-533.

Szoka, P.R., Achreiber, A.B., Chan, H. and Murthy, J. (1986) DNA. 5:11-20.

Tan, N.S., Ho, B. and Ding, J.L.(2000a) FASEB. J. 14:859-870.

Tan, N.S., Ng, M.L., Yau, Y.H., Chong, P.K., Ho, B. and Ding, J.L.(2000b). FASEB. J. 14:1801-1813.